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Review

Control of eukaryotic membrane fusion by N-terminal domains of SNARE proteins

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Abstract

SNARE proteins function at the center of membrane fusion reactions by forming complexes with each other via their coiled-coil domains. Several SNAREs have N-terminal domains (NTDs) that precede the coiled-coil domain and have critical functions in regulating the fusion cascade. This review will highlight recent findings on NTDs of syntaxins, the longin domain of VAMP proteins and SNAP-23/25 homologues in yeast. Biochemical and genetic experiments as well as the resolution of several NMR and crystal structures of SNARE NTDs shed light on their diverse function.

Keywords: SNARE; N-terminal domain; Syntaxin; Longin; SM protein; Vam7

1. Overview

SNARE proteins are key players in eukaryotic membrane fusion [1–3]. Most SNAREs have a C-terminal transmembrane domain and a large cytosolic domain. Once a vesicle docks to its target organelle, SNAREs localized to both membranes form defined complexes with each other via their membrane-proximal coiled-coil, or SNARE, domains [4,5]. Reconstitution of SNAREs into liposomes suggested that only specific complexes are capable of fusing membranes [6]. However, it is still a matter of debate whether such an activity is sufficient to account for the precise and fast fusion necessary within cells [7,8]. In addition to coiled-coil domains, several SNAREs have amino (N)-terminal extensions that can fold independently. This review will focus on our current understanding of these SNARE *appendices* as key elements that regulate and coordinate membrane fusion reactions.

Recently, Misura et al. [9] presented a comprehensive alignment of the N-terminal domains (NTDs) of SNAREs that were grouped by their SNARE motif. From this, it becomes apparent that all syntaxins have similar overall structures. Among VAMP/synaptobrevin homologues, the longin group—Sec22, Ykt6 and VAMP7—also have a general similarity [10]. However, SNAREs homologous to SNAP-25 C- or N-terminal helices are more divergent, and few structures are known.

Here, we will focus on the structure and function of characterized NTDs. First, we will discuss syntaxins, all of which have elongated amino-terminal domains consisting of three helices. The second part will focus on v/R-SNARE homologues. This group consists of two classes: longins have a folded N-terminus [10], whereas synaptobrevin-like v-SNAREs have a short and probably unfolded N-terminus. In the last part we will discuss a few exceptional cases, focusing on the vacuolar SNAP-23 homologue Vam7, which contains a phosphoinositide-binding PX domain. A close examination of Vam7 reveals evolutionary principles that may explain its unique character. A summary of the domains and the nomenclature of SNAREs are given in Table 1.

2. The N-terminal sandwich of syntaxin-like t-SNAREs

The NTDs of syntaxin-like t-SNAREs have been studied extensively over the last years. A wealth of structural and biochemical data is now available that illuminates the N terminus as a regulatory unit. Generally, the NTD of

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Table 1
SNAREs with characterized NTDs

Class	SNARE	SM protein	Compartment	Interactions	PDB and ref.	Structure of N-terminus
Syntaxin	Sed5 /Syntaxin 5	Sly1	Golgi	Binding to	1MSQ; [37]	
(t-SNARE	Ufe1 / Syntaxin 18	Sly1	ER	SM via	**; [33]	
Q-SNARE)	Tlg2 / Syntaxin 16	Vps45	TGN, EE	N-terminal peptide	**; [34]	Three helical bundle, does *not* bind coiled-coil domain
	Pep12	Vps45	LE	unknown	**; [34]	
	Vam3	Vps33	Vacuole	Indirect binding as part of a complex	1HS7; [44]	
	Syntaxin 6*	?		?	1LVF; [9]	
	mVti1b*	?		?	**; [31]	
	Sso1, Sso2	Sec1	PM	Binding of Sec1 to SNARE complex	1F10; [25]	Three helical bundle, binds to coiled-coil domain
	Syntaxin 1A	Munc18-1	PM	1:1 complex	1EZ3; 1BRO [22;24]	
	Syntaxin 7	?	LE	?	** ; [30]	
Longin	Sec22b		ER / Golgi	?	1lFQ; [58]	Profilin-like fold
(VAMP,	Ykt6		Golgi, vacuole	?	1H8M; [59]	
R-SNARE	Ti-VAMP		dendrites	?	**; [63]	?
v-SNARE)						
exceptions	Vam7	/	Vacuole	PtIns-3-P	1KMD; [74]	PX domain

Yeast proteins are listed first and have three letter abbreviations and a number. Only biochemically and structurally characterized proteins are listed. Abbreviations are as follows: PM (plasma membrane), ER (endoplasmic reticulum), EE (early endosome), LE (late endosome), TGN (trans-Golgi network), EE (early endosome). An asterisk (*) on mVti1b and on syntaxin 6 indicates that these proteins do not belong formally to the syntaxin group, though they have structural similarities. (**) indicates supporting biochemical, genetic or NMR data if structures have not been solved. Nomenclature of SNAREs according to Rothman (*v*esicle-and *t*arget organelle SNAREs; [1]) and Fasshauer et al. (based on zero layer of SNARE motif in crystal structure and computer-based alignments [89,90]: central arginine = R-SNARE, central glutamine = Q-SNARE; [91]) is in brackets. Additional classifications of the Q-SNAREs into the syntaxin group, the S25N and S25C group (i.e. SNAP-25 N or C-terminal helix) have been suggested [9].

syntaxin-like t-SNAREs consists of a short unstructured N-terminal peptide sequence followed by the H_{abc} domain (Fig. 1). The H_{abc} domain folds into a three-helix bundle with a left-handed twist. The plasma membrane syntaxins, neuronal syntaxin1A and yeast Sso1, have an additional groove that can accommodate the SNARE motif and convert the protein to a closed conformation. The NTD is required for several regulatory reactions, including the variable interaction with Sec1/Munc18 proteins (SM proteins). We will discuss three groups in this context: (1)

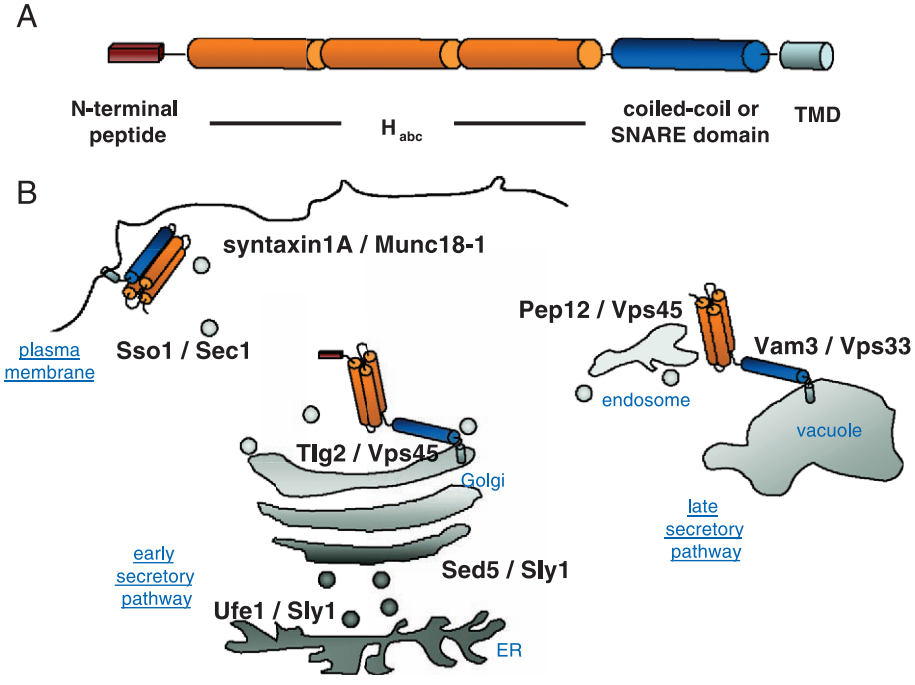


Fig. 1. Localization of the three groups of syntaxin-like t-SNAREs within cells. (A) Domains are distinguished by colors: N-terminal peptide (red), H_{abc} region (orange), coiled-coil domain (blue) and transmembrane domain (TMD; light blue). (B) Three classes of syntaxin–SM interactions within the secretory pathway. For details see text.

syntaxins that can adopt a closed conformation, (2) syntaxins that bind to SM proteins via an N-terminal peptide, and (3) syntaxins that recognize the SM protein in the context of a larger protein complex.

2.1. The closed syntaxins

The importance of the H_{abc} domain initially became apparent with the characterization of syntaxin1A, the t-SNARE of neuronal presynaptic terminals. Upon isolation, syntaxin1A is in the closed conformation, in which the NTD is bound to the coiled-coil SNARE motif. Munc18-1, a SM protein of the synapse, binds tightly to the closed form of syntaxin1A in a 1:1 complex that precludes binding of the presynaptic SNAREs SNAP-25 and synaptobrevin, as well as α-SNAP [11–14]. When Munc18-1 is bound to syntaxin1A, synaptic *trans*-SNARE complexes cannot form. On the other hand, Munc18-1 is essential, and synapses are absolutely silent when prepared from mice embryos lacking Munc18-1 [15]. Another neuronal protein, Munc13/unc13, binds the N-terminus of syntaxin1A specifically [16]. Munc13 is an essential, multi-domain protein, which contains a phorbol-ester binding C1 domain that is important in the regulation of vesicle priming by interacting with diacylglycerol [17–19]. It is possible that Munc18-1 and Munc13 cooperate to protect and guide syntaxin1A prior to the formation of *trans*-SNARE complexes: (i) Munc18-1 would first bind syntaxin1A in the closed form. (ii) This is then converted to the open form by an unknown mechanism, which is stabilized by Munc13. (iii) The open syntaxin1A would then participate in *trans*-SNARE complexes that form prior to membrane fusion. This model is supported by the observation that syntaxin1A mutants that are constitutively open do not bind Munc18-1 and inhibit exocytosis [20]. Interestingly, in *C. elegans* a requirement for Unc13 becomes dispensable if syntaxin1A is present as a constitutively open mutant [21]. However, a sequential reaction of Munc18-1 and Munc13 has not yet been shown, and the transition of the closed to the open form of syntaxin1A awaits further analysis.

There are strong similarities between syntaxin NTDs. Both syntaxin1A and its homologue at the yeast plasma membrane, Sso1, have a groove in their NTD that is adapted to accommodate the coiled-coil motif (Fig. 2A; Refs. [22–24]). Assembly of SNARE complexes in vitro is accelerated 2000-fold in the absence of the Sso1 NTD [25], and fusion of liposomes carrying synaptic SNARE proteins is promoted if the syntaxin1A NTD is removed [26]. This indicates that the closed conformation of syntaxin1A and Sso1 is inhibitory for fusion, but in both cases it seems that the closed conformation is also an important intermediate that prevents fast SNARE pairing. Sso1 requires the N-terminus for yeast viability [23] even though a constitutively open Sso1 can bind efficiently to Sec9, a yeast SNAP-25 homolog. If Sec9 is overexpressed in this mutant, the cells become sick, indicating that the syntaxin's ability to form

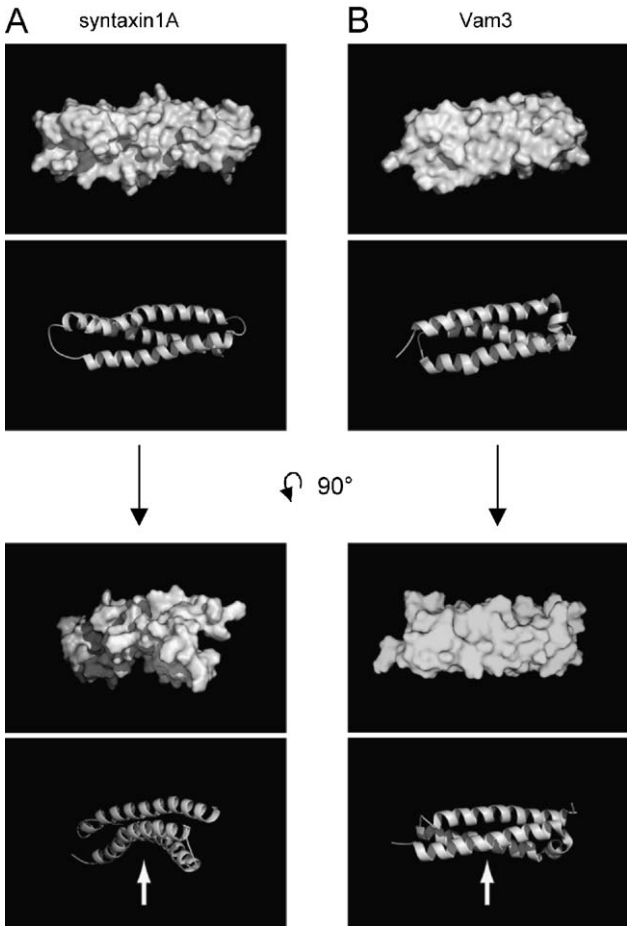


Fig. 2. Comparison of syntaxin1A (A) and Vam3 (B). Top structures were rotated by 90° to show the groove within the syntaxin N-terminus (PDB accession numbers 1EZ3 and 1HS7 [22,44]). Both ribbon and surface structures are displayed. All structure figures were generated with PyMOL.

a closed conformation is essential for productive SNARE complex formation [27]. Despite the similarity between syntaxin1A and Sso1, proteins that bind the N-terminus of Sso1 have yet not been identified. Furthermore, what appeared to be the paradigm for binding of syntaxins to SM proteins—the syntaxin1A/Munc18-1 interaction—turned out to be an exceptional case. Sso1 does not form a binary complex with its SM protein Sec1, rather, they only interact in the context of the SNARE complex [28]. Binding of Sec1 to Sso1 in vitro is very poor, in contrast to the tight interaction of Munc18-1 and syntaxin1A [13,14,28]. Furthermore, though Sec1 apparently binds Sso1 in the SNARE complex, it actually has not yet been tested whether Sec1 still binds to the SNARE complex if Sso1 lacks its N-terminus. Since it accumulates at sites of secretion [28], Sec1 binding may enhance exocytosis by altering the *trans*-SNARE complex [29]. Fast exocytosis in the synapse seems to require guidance of syntaxin1A by its direct binding to Munc18-1. Whether this is a very specialized case or could be similar to Sec1's enhancement of fusion at the yeast plasma membrane remains to be seen. An in vitro fusion

assay to unravel the sequential events in exocytosis in yeast could become helpful to clarify the role of Sec1.

Recently, it was shown that syntaxin7, a syntaxin of the late endosome, also adopts a closed conformation [30]. Syntaxin7 forms a SNARE complex with Vti1b, syntaxin8 and endobrevin [31,32]. Removal of the NTD of syntaxin7 accelerates SNARE complex assembly in vitro sevenfold. However, known SM proteins do not bind the N-terminus of syntaxin7 in a two-hybrid assay [33]. A function of syntaxin7's NTD and its role in endosome fusion awaits further analysis.

2.2. The open syntaxins with an N-terminal peptide motif required for SM binding

Within the secretory pathway, between the endoplasmic reticulum (ER) and the endosome, several syntaxins interact with SM proteins via an unexpected mechanism. These syntaxins have amino-terminal domains comparable to syntaxin1A, but which lack the central groove required for an interaction with the coiled-coil domain [9,33,34]. They also have an unstructured ~30-amino-acid peptide stretch, which precedes the three-helix bundle of the H_{abc} domain. It is now clear that some SM proteins recognize and bind this peptide specifically. At the ER and Golgi, the SM protein Sly1 binds to the syntaxins Ufe1 and Sed5, and the endosomal SM protein Vps45 interacts with Tlg2/syntaxin16 [33,34]. Binding of SM proteins to the N-terminal peptide has functional consequences. At the Golgi, the Sly1 interaction with Sed5 occurs in the context of the SNARE complex and contributes to the specificity of SNARE complex assembly [35,36]. Structural studies on the interaction of Sly1 with Sed5 were recently published [37,38]. Strikingly, Sly1 binds to the Sed5 N-terminal peptide via an autonomously folded N-terminal part. This interaction is distinct from the previously discussed binding of Munc18-1 to syntaxin1A, since it occurs on the opposite face of the SM protein [37,38]. At the endosome, Vps45 binds and stabilizes Tlg2 and is necessary to permit SNARE complex assembly in vivo [39,40]. Structural considerations suggest that Vps45 binds to Tlg2 like Sly1 to Sed5 [37,38]. Interestingly, a Tlg2 mutant lacking the NTD can partially bypass the requirement for Vps45 [40]. These data suggest that binding of SM proteins to the N-terminal peptide activates and chaperones the SNARE to the *trans*-SNARE complex[1].

2.3. The late secretory pathway syntaxins and complexed SM proteins

In the late endocytic pathway, the interactions between the NTD of the syntaxins Vam3 and Pep12 with the SM proteins Vps33 and Vps45, respectively, appears to be indirect. In contrast to all previously discussed SM proteins, Vps33 has not been found as a monomer but as part of a large complex, the Class C Vps or HOPS complex [41–43]. Vam3 contains a shorter N-terminal three-helix bundle without a syntaxin1A-like groove (Fig. 2B; Ref. [44]). The N-terminus of Vam3 is not essential, but mutant vacuoles fuse with markedly reduced efficiency [45]. This is probably due to its poor interaction with the Vps33-containing Class C Vps/HOPS (homotypic fusion protein sorting) complex [45], an essential docking factor [46,47]. Based on in vivo associations, it has been suggested that the HOPS complex binds exclusively to unpaired Vam3 [48]. Even though it remains to be shown when such an intermediate occurs during the vacuole fusion reaction, it appears that the interaction of Vam3 and the HOPS complex is required to allow a coordinated transition from priming to docking. However, not all studies agree that the NTD of Vam3 is essential for this. It has been shown that antibodies to the Vam3 N-terminus do not block vacuole fusion [49]. This is a difficult argument, though, as it is unclear if the antibody recognized its membrane bound target. In the same study, the reduction in vacuole fusion with truncated Vam3 was not as significant as in our studies. Furthermore, the coiled-coil domain of Vam3 can also bind monomeric Vps33 [44], but it is uncertain whether this interaction also occurs in vivo, as a function of monomeric Vps33 has not yet been reported. Thus, even though the biochemical evidence for a role of the NTD of Vam3 is strong, unraveling an accurate picture will require further studies.

Little is known about the function of the amino terminus of the endosomal syntaxin Pep12. Like the Vam3 N-terminus, it contains a folded three-helix bundle and does not adopt a closed conformation, but lacks the N-terminal peptide found in Tlg2 [34]. Pep12 has been identified in a complex containing the SM protein Vps45, the FYVE-domain protein Vac1 (a tethering factor similar to mammalian EEA1 and rabenosyn) and the Rab GTPase Vps21 [39,50–53], but a direct binding of Vps45 to Pep12 has not been observed [33]. A close characterization of this complex is again hampered by the lack of an in vitro assay. Thus, it remains to be established whether and how the N terminus of Pep12 binds to this complex and at which time during fusion the complex forms. Previous analysis of vacuolar and endosomal SNARE complexes in the *sec18-1* temperature-sensitive mutant (Sec18 is the ATPase necessary to disassemble SNARE complexes) indicated that complexes observed in vivo may not correspond to intermediates of a fusion reaction but are most likely the result of a completed fusion reaction [54,55].

The intense efforts to unravel structures and functions of the NTDs of syntaxin-like t-SNAREs allow us to deduce some common themes. All syntaxins characterized to date contain an N-terminal three-helix bundle. The possibility of forming a closed conformation by binding to the SNARE motif is reserved for the plasma membrane syntaxins. SM proteins are the major binding partners of the NTD. The

[1] Differential binding of SM protein Vps45 to Tlg2 has recently suggested by N.J. Bryant and D.E. James, J. Cell Biol. 161 (2003) 691–696.

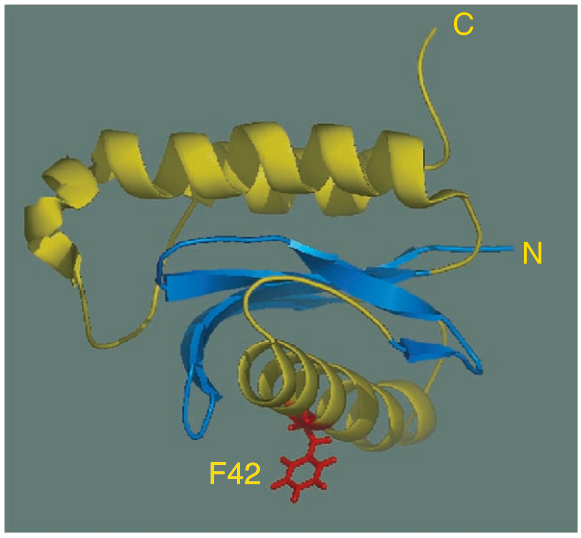
recognition site of SM proteins can include the H_{abc} domain (Sso1 and syntaxin1A), but more common is the binding to the unstructured N-terminal peptide. It appears as if all SM proteins that show a direct binding to the syntaxin are required to chaperone activated t-SNAREs. The intermediate may be required to allow specific assembly of *trans*-SNARE complexes [35], prevent degradation of the unstable intermediate [40] or trigger fast exocytosis [8]. So far, it is uncertain whether such a mechanism also functions at the late endosome or vacuole, where SM proteins act in the context of larger protein complexes. Specific interactions between the SM proteins Vps45 and Vps33 and the NTD of Pep12 and Vam3, respectively, have not been discovered. One provocative hypothesis is that the NTD of syntaxins functions as an anchor to guide SM proteins to their site of action.

3. Longin domains

In the late 1980s, VAMP [56] and its homologue synaptobrevin [57] were isolated as vesicle-associated proteins from synapses and predicted to be crucial for vesicular transport. Today they define a group of SNARE proteins that share sequence homology, in particular the highly conserved arginine, within their coiled-coil/SNARE motifs. They are also known as v-SNAREs or R-SNAREs.

The amino termini of proteins in the VAMP/synaptobrevin family are diverse, ranging in length from a few (synaptobrevin) up to 150 residues (Sec22). It was only recently recognized that some of the long NTDs (~ 150 aa) share significant sequence homology [10]. This resulted in further classification of these VAMP/synaptobrevins as longins, allowing the others with less conserved N-termini to be referred to as brevins. Information on the N-termini of brevins is scarce and awaits further analysis. Here, we will focus on recent studies on the longin family. Bioinformatic analysis predicted that longin domains share the same secondary structure [10]. This was confirmed by two studies, which solved the crystal structures of the NTDs of the R-SNAREs mouse Sec22b [58] and yeast Ykt6 [59]. Both longin domains were shown to adopt the same fold, a globular domain with a five-stranded β-sheet that is sandwiched by an α-helix on one side and two α-helices on the other (Fig. 3A). The same arrangement is also found in the actin-regulating protein profilin [60], GAF/PAS regulatory modules [61] and the tethering factor SEDL [62].

Surprisingly, while longins are conserved in all eukaryotes, sequence similarity searches suggest that other VAMP/synaptobrevins are lacking in plants [10]. This indicates that the R-SNARE motif of longins can substitute for that of brevins. Since longins are the only VAMP/synaptobrevin-type found in all eukaryotes, their NTD appears to be essential.



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Ykt6     MRIYYIGVFRSGGEKALELSEVKDLSQFGFFERSSVGQFMTFFAETVASRTGA...GERQ
Sec22    .SVLLTXIARVAD..GLPLAASXQEDEQ....SGRDLQQYQSQAKQL.FRKLneqsPTRC

Ykt6     SIEEGNYIGHVYARSEGICGVLITDKQYPVRPAYTLLNKILDEYLVAHPKEEWADVTETN
Sec22    TLEAGAXTFHYIIE.QGVCYLVLCEAAFPKKLAFAYLEDLHSEFDEQH.GKKVPTVSRPY

Ykt6     .DALKMKQLDTYISKYQDPSQADA
Sec22    sFIEFDTFIQKTKKLYI.......
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Fig. 3. Structure of the NTDs of longins. (A) Ykt6 structure (PDB accession number 1H8M, [59]). F42 that is essential for autoinhibition of Ykt6 [59] is highlighted in red. β-sheets are in blue, α-helices in yellow. (B) DALI structural alignment of the NTDs of Sec22b and Ykt6. Identical residues are indicated in green. Note that the Sec22 sequence was modified by the DALI program: X corresponds to M (methionine).

What is the function of this domain? Two studies, addressing the longin domains of Ti-VAMP (VAMP7) and Ykt6, suggest an autoinhibitory mechanism that is reminiscent of syntaxins that can adopt the closed conformation, as discussed in the previous section. Galli and coworkers showed that expression of an N-terminally deleted Ti-VAMP supports neurite outgrowth of neurons in primary culture, whereas overexpression of the NTD of Ti-VAMP has the opposite effect [63]. This was the first indication that longin domains exert a regulatory function. Zhang and coworkers demonstrated that the longin domain of Ykt6 contains a hydrophobic patch with which it can bind its SNARE motif [59]. The mutation of a crucial residue within this patch, F42E, was sufficient to abrogate this interaction in an in vitro binding assay. Since the same mutation conferred a loss-of-function phenotype in vivo, an autoinhibitory mechanism of the NTD was suggested. In contrast, for Sec22 an interaction between the longin domain and the SNARE motif was shown to be unlikely [58]. Interestingly, like Ykt6, Sec22 also contains a hydrophobic patch in a similar spatial orientation, but at the position of the crucial Phe in Ykt6, Sec22 contains a serine (Fig. 3B). Since the F42E mutation in Ykt6 prevents an interaction between longin domain and SNARE motif, the structural comparison nicely confirms the negative binding results for Sec22.

Based on these data, the autoinhibitory function of Ykt6 and Ti-VAMP NTDs seems not to be a unifying feature of longins. As a consequence, the same fold, i.e. the profilin-like domain, can adopt different functions. Significantly, the same fold in profilin—without any sequence homolog to longins—has an entirely different function. Based on a structural comparison between Sec22's longin domain and profilin, Sec22 is unlikely to mediate functions of profilin (like the interaction with proline-rich sequences, actin or phosphoinositide phosphates) [58]. An analogous structural comparison between Ykt6 and profilin suggests the same (Fig. 3).

Recently, a structural study on the tethering factor SEDL revealed a fold shared with Sec22-Ykt6, but also with no sequence homology [62]. Is the longin/profilin-fold then merely a structural scaffold that allows a variety of functions depending on its environment? Or, does the special sequence conservation of the longin domain encode a unifying function that remains to be identified? While this issue has not been resolved, interesting players regarding the function of the longin domain are the mammalian isoforms Sec22a and Sec22c [64–66]. Both are unlikely to be SNARE proteins since their C-terminal extensions are not predicted to form coiled-coil domains [67]. Their existence suggests a function for the longin domain that exceeds the role of a SNARE-regulating domain, as it may even function without the SNARE motif. Whether or not these two proteins are involved in membrane fusion and whether the longin domain exerts additional functions independent of fusion remains to be clarified.

4. Unusual NTDs

Structural and functional analyses have revealed conserved features of the syntaxin and the longin families. In the last part, we will discuss three yeast proteins of the SNAP-25/23 family, which also have N-terminal extensions: the vacuolar SNARE Vam7, and the plasma membrane SNARE Sec9 and its homolog Spo20. In contrast to all previously discussed SNAREs that are bound to the membrane via a C-terminal transmembrane domain or lipid anchor, Vam7, Sec9 and Spo20 contain neither. They share a similar coiled-coil domain, but the functions of their N-termini are divergent. In this part we will focus primarily on the vacuolar SNARE Vam7 and briefly discuss illuminating findings on Sec9 and Spo20.

4.1. Vam7 function on the vacuole

The vacuolar SNAP-23 homologue Vam7 belongs to the t/Q-SNARE family and is part of the vacuolar SNARE complex. Vam7 is required for vacuole fusion and maintenance of vacuolar integrity since vacuoles are fragmented in the absence of Vam7 [48,68,69].

Vam7's NTD contains a PX domain. PX domains (named after the NADPH oxidase subunits $p40^{phox}$ and $p47^{phox}$) were identified as novel phosphatidylinositol phosphate (PtdIns-P) binding proteins, most of them having a preference for PtdIns-3-P [70,71]. Several yeast proteins with PX domains, including Vam7, bind to PtdIns-3-P [72,73]. The PX domain consists of a module of three β-strands and three to four α-helices that binds to the phosphoinositide (Fig. 4; Ref. [74]). Binding to phosphoinositides may insert part of the PX domain into the lipid bilayer, similar to the lipid insertion seen for the FYVE domain (M. Overduin, personal communication). So far, it is unclear whether PX domains also undergo specific protein–protein interactions. The binding affinity of the PX domain to membranes has not been determined precisely, but Vam7 resists membrane extraction by carbonate or urea [69]. It is likely that the interaction of the coiled-coil domains of Vam7 and Vam3 contributes to the tight membrane association.

In yeast, Vam7 is found both on the vacuole and in the cytosol. Inhibition of Sec18p in vivo shifts most of Vam7 to the vacuole [68]. We could show that Vam7 moves between the vacuole membrane and the cytosol [75]: during ATP/Sec18-dependent priming, up to 90% of Vam7 gets released from the vacuole membrane into the cytosol as a monomer. The released Vam7 is a functional intermediate, as it can rescue fusion of vacuoles depleted of Vam7. In agreement with Cheever et al. [72], we found that Vam7 required its PX domain for this rescue. Since the C-terminal coiled-coil domain of Vam7 and a Vam7 protein mutated in its PX domain do not rescue fusion, it is likely that association of the PX domain to a phosphoinositide target is a prerequisite for the migration of Vam7 into the fusion site. We do not know whether PtdIns-3-P is sufficient to allow Vam7

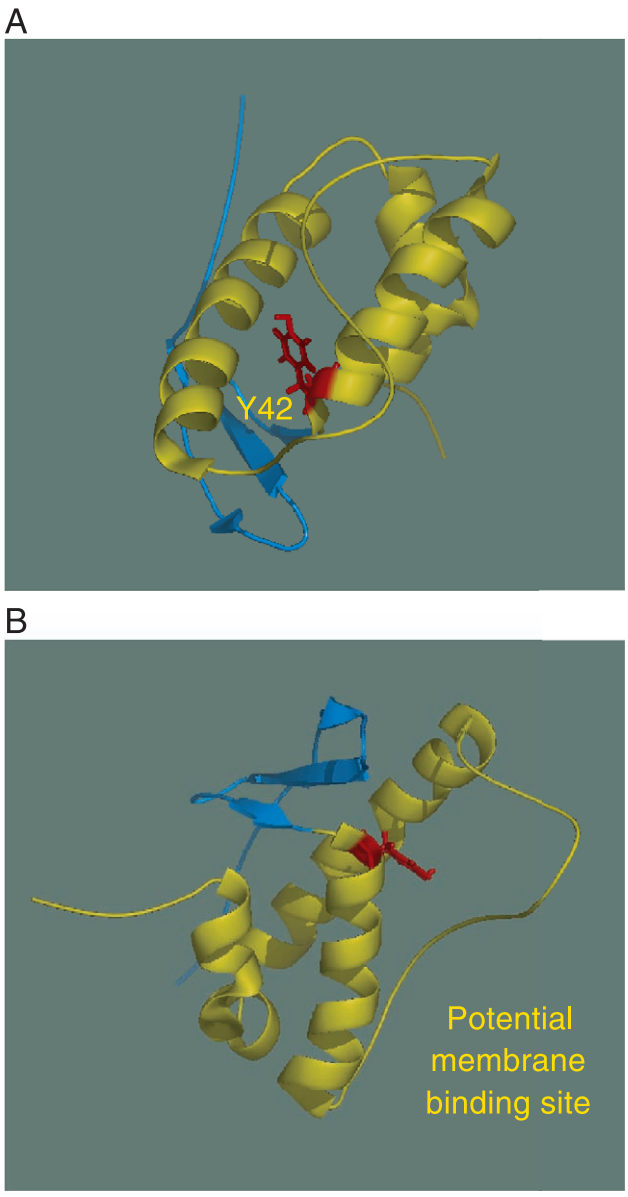

Fig. 4. Structure of the PX domain of Vam7 (PDB accession number 1KMD; [74]). The critical Y42 [68,72] that may bind directly to PtdIns-3-P is shown in red. Color code as in Fig. 3.

binding. Vam7 cannot bind to vacuoles that were not primed. This indicates that either all PtdIns-3-Ps are occupied, have not been generated, or binding can only occur when a Vam3 protein–protein interaction site is available. Thus, even though we know the major lipid-binding site of Vam7, we are far from understanding the regulation of Vam7 movement between the cytosol and the vacuole.

4.2. Phosphoinositides, acylation and SNARE protein function

Phosphoinositides are involved in a broad range of cellular processes including membrane trafficking along the secretory pathway, the cell cycle and autophagy. Several conserved domains bind to PtdIns phosphates, such as the PH (pleckstrin homology) domain, the ENTH (epsin N-terminal homology) domain, the FYVE (Fab1p, YOTB, Vac1 and EEA1) domain, the FERM domain (band 4.1, ezrin, radixin and moesin) and the PX domain [70,76]. Several PX domain-containing proteins have functions in protein sorting, for example the yeast proteins Vps5 and Vps17. Both proteins are part of the retromer complex that is required for transport of proteins from the endosome to the Golgi [77–80]. Interestingly, coiled-coil domains in Vps5 and Vps17 flank the PX domain C-terminally, similar to the domain arrangement of Vam7. It is likely that such an orientation is determined by the binding mode of the PX domain to lipid membranes. So far, it is not clear why Vam7 is the only known SNARE (with the exception of two potential homologues in the yeasts *S. pombe* and *C. albicans*), which requires a PX domain normally found in proteins essential for sorting along the secretory pathway. We hypothesize that the PX domain of Vam7 represents a phosphoinositide-binding module that is essential for each membrane fusion machinery. On other organelle membranes along the secretory pathway, these modules are found in tethering factors like mammalian EEA1 or yeast Vac1/rabenosyn5, raising the possibility that lipid domains have a role at the fusion site. Indications for such a domain are few. Ergosterol and PtdIns-4,5-P_2 are required for vacuole fusion [81,82], but any connection to the function of the Vam7 PX domain has not yet been established. To date, Vam7 is the only PtdIns phosphate-binding protein required for vacuole fusion, although it is likely that PtdIns-4,5-P_2 also has a protein target [82]. Thus, it seems that for each membrane fusion reaction, at least one protein has a key function to guide and sort others to defined lipid domains. Proteins required for PtdIns-P synthesis and turnover could therefore have key functions in membrane-fusion reactions.

The mammalian SNAREs SNAP-23 and, to some extent, SNAP-25 show homology in one of their two SNARE motifs to the Vam7 coiled-coil domain. Both proteins lack TMDs, but are anchored to the membrane by palmitoylation [83,84]. Like Vam7, SNAP-23 cycles between membranes and the cytosol [85,86], a movement possibly associated with a palmitoylation/depalmitoylation switch. We speculate that Vam7 function as a mobile SNARE has been preserved during evolution by transferring the PtdIns phosphate binding to tethering factors and anchoring the SNARE motif by palmitoylation.

4.3. Determining specificity by the N-terminus: Sec9 and Spo20

During exocytosis, Sec9, the SNAP-25 homologue at the yeast plasma membrane, acts together with the syntaxin-like t-SNARE Sso1. During spore formation, Sec9 gets replaced by Spo20, which is 40% homologous to Sec9 and essential for sporulation [87]. Interestingly,

despite the substantial overall homology of these proteins, the NTDs appear to have distinct functions. Replacement of the Sec9 N-terminus by the one of Spo20 inhibits vegetative growth, while the Sec9 amino-terminus can be removed and does not affect fusion [88]. These studies underline the importance of these NTDs, and it will be interesting to see what structural data on these proteins will contribute to understanding what seem like significant functional differences.

5. Concluding remarks

Numerous structural, genetic and biochemical studies have provided insight into the role of the NTDs of SNARE proteins. The N-terminal three helical bundle of syntaxin-like t-SNAREs appears to be a conserved feature of this protein group, as does the profilin like fold for longins. There are several challenges for future research. The conservation of Sec1 proteins and their relation to the NTDs of syntaxin-like t-SNAREs is obvious in some cases, but the common function of Sec1 proteins remains to be solved. Equally, the conservation of the longin domain of Sec22, Ykt6 and Ti-VAMP is striking, though it is likely that their function will exceed the autoinhibitory role described for Ykt6 and Ti-VAMP. Structures of the NTDs of other v-SNAREs are not available, nor is it known whether they form autonomous domains. Future studies will certainly unravel many of these questions and provide unexpected insight into the emerging picture of SNARE protein function beyond SNARE motifs.

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